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(54) Title: DNA MOLECULES ENCODING INDUCIBLE PLANT PROMOTERS AND TOMATO ADH2 ENZYME

(57) Abstract

An isolated DNA molecule comprising a nucleotide sequence encoding an inducible soft fruit promoter, particularly the alcohol dehydrogenase 2 promoter from tomato, is described. Isolated DNA molecules encoding the alcohol dehydrogenase 2 enzyme are also disclosed.

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DNA MOLECULES ENCODING INDUCIBLE PLANT PROMOTERS AND TOMATO ADH2 ENZYME

This invention relates to inducible plant promoter DNA sequences, DNA sequences encoding tomato alcohol dehydrogenase 2 (ADH2) enzyme, and hybrid DNA molecules incorporating such sequences. In a particular application of the invention, these hybrid DNA molecules are used to transform plants to enable the control of the condition and quality of fruit.

In the soft fruit industry (e.g. strawberries, peaches, plums and tomatoes), substantial losses are incurred during transport, storage and marketing because of the susceptibility of softer fruit to mechanical damage and invasion by microorganisms. To limit such losses, plant breeders have selected lines which are less soft, and for many fruits firm cultivars now dominate national and international trade. Unfortunately, while marketing losses may be lower when fruit is less soft, the firmer lines may have reduced market appeal and also tend to be deficient in flavour.

In some fruits, such as tomatoes, avocados and peaches, softening arises from the degradation of cell wall components by enzymes deposited in response to signals associated with ripening. Ripening signals typically include ethylene and the fruit response to ethylene and other signals can be strongly affected by temperature, and by oxygen and carbon dioxide concentrations. In firm fruit cultivars, the response to ripening signals has usually been damped so that less enzyme is produced; softening is correspondingly protracted and unless the signal response can be enhanced, by the time the fruits are acceptably soft the tissue is often senescent and lacking in flavour and aroma.

In higher organisms, structural genes, including those involved in softening and the development of flavour

and aroma in fruit, are switched on by DNA sequences known as promoters, which recognise internal signals and "promote" transcription of the adjacent functional genes. Promoters which respond to oxygen stress or to heat shock have been characterised in plants, for example alcohol dehydrogenase (ADH) accumulates in plants after 5 to 10 hours of oxygen stress, and heat shock proteins can be detected after only brief periods of high temperature. In each case the new chemical entities are produced through the response of promoters to environmental signals. Thus, these kinds of inducible promoters permit the construction of hybrid DNA molecules in which a structural gene encoding, for example, a polypeptide involved in fruit softening, is brought under the control of an inducible promoter sequence, so that the structural gene is transcribed when the promoter is subjected to an activating signal.

Accordingly, in a first aspect the present invention provides an isolated DNA molecule comprising a nucleotide sequence encoding a soft fruit promoter or functional portion thereof, wherein said promoter or functional portion thereof is characterised in that it can be activated by environmental agents or conditions and/or is activated, or primarily activated, during a late stage of normal soft fruit ripening.

Preferably the soft fruit promoter or functional portion thereof is activated thermally, chemically or by light. In the case of chemical activation, preferred promoters and functional portions thereof will be responsive to particular levels of gases such as oxygen, carbon dioxide or carbon monoxide. Alternatively, chemical activation may be achieved through exposure to some organic acids.

The soft fruit promoter or functional portion thereof may be isolated from grapes, strawberries, peaches, plums

or tomatoes. Preferably the soft fruit promoter or functional portion thereof is activated by environmental agents or conditions, and is further characterised by also being activated, or primarily activated, during a late stage of normal ripening. For example, for tomato, during the period of ripening following 5-6 days after ripening-onset. More preferably, the promoter or functional portion thereof is the tomato alcohol dehydrogenase 2 (ADH2) promoter.

Thus, in a second aspect the present invention provides an isolated DNA molecule comprising a nucleotide sequence encoding, or substantially homologous to, the tomato ADH2 promoter or a functional portion thereof.

The term "substantially homologous" as used herein in relation to the nucleotide sequence encoding the tomato ADH2 promoter or functional portion thereof, is intended to encompass nucleotide sequences with sufficient homology to hybridise to the nucleotide sequence encoding the tomato ADH2 promoter or functional portion thereof under medium to high stringency conditions (Maniatis et al. in "Molecular Cloning - a laboratory manual", Cold Spring Harbor Laboratory 1982). Such substantially homologous nucleotide sequences may contain single or multiple nucleotide substitutions and/or deletions and/or additions thereto.

Most preferably, the isolated DNA molecule comprises a nucleotide sequence substantially corresponding to that shown in Table 1 from residue -942 to -1.

In order to isolate the tomato ADH2 promoter, it was necessary to first isolate the tomato ADH2 encoding sequence. The present inventors have thus isolated a cDNA (see Table 1) which was used to prepare hybridisation probes for isolation of a genomic fragment encoding ADH2 and containing the ADH2 promoter sequence.

In a third aspect, the present invention provides an

isolated DNA molecule comprising a nucleotide sequence encoding a plant promoter obtained from a genomic fragment isolated using a labelled nucleic acid probe comprising a nucleotide sequence substantially corresponding to the cDNA sequence or a portion thereof shown in Table 1.

The probe may be DNA or RNA transcripts.

DNA molecules according to any of the preceding aspects may further comprise nucleotide sequences encoding peptides or polypeptides expressibly linked to the promoter sequence. The peptide or polypeptide may be involved in fruit softening, flavour, colour or aroma, for example polygalacturonase or its subunits, pectin methyl esterase, xyloglucanase or other beta-1,4-glucanases, glycosidases, beta-galactosidase, alcohol dehydrogenase or lipoxygenase. Further, ripe fruit are more susceptible than unripe fruit to fungal invasion. Thus the peptide or polypeptide encoding sequence may encode fungal resistance agents (e.g. chitinase, beta-1,3-glucanase) or other plant pathogen resistance agents.

The peptide or polypeptide encoding sequence or a portion thereof (e.g. a 20-50 nucleotide portion), may be linked in the opposite orientation for expression in a 3' to 5' direction such that antisense RNA is produced. Ribozymes for cleaving mRNA's encoding the peptide or polypeptide may be produced in a similar manner. In this case, the oppositely orientated nucleotide sequences provide the ribozymes with specificity and a further nucleotide sequence encodes a catalytic domain for cleavage of mRNA. Suitable catalytic domains include hammerheads, hairpins, delta-virus elements, ribosome RNA introns and their derivatives. Further information regarding the design of ribozymes can be found in Haseloff, J. & Gerlach, W.L. (1988) Nature Vol. 334:585, and Kruger, K. et al. (1982) Cell Vol. 31, 147; International Patent Application No. WO 88/04300, US

4,987,071 and US Patent No. 5,254,678. The disclosure of each of these references is incorporated herein by reference.

Further, DNA molecules according to the invention may include enhancer elements (e.g. from the octapine synthase gene of Agrobacterium tumefaciens - Ellis, J.G. et al., 1987 EMBO J. 6:11-16), or multiple copies of an anaerobic response element (ARE) (Olive, M.R. et al., 1990 Plant Mol. Biol. 15: 593-604), or intron sequences.

The tomato ADH2 enzyme is believed to be involved in fruit ripening by metabolising hexanols/als and methoxybutanols/als, which are important fruit flavour volatiles. By modifying the activities and/or abundance, in ripening fruit, of the enzymes involved in production of flavour volatiles, it is possible to increase their production of flavour volatiles independently of the rate of fruit softening and thereby enhance development of flavour in the fruit. Thus, transgenic plants with modified ADH2 levels may have direct commercial value and provide valuable stocks for breeding programs.

Thus, in a fourth aspect, the present invention provides an isolated DNA molecule comprising a nucleotide sequence encoding tomato ADH2 or a fruit ripening and/or aroma/flavour-affecting portion thereof.

Preferably, the isolated DNA molecule comprises a nucleotide sequence substantially corresponding to the cDNA sequence shown in Table 1 or the genomic sequence from residue 1-2175 shown in Table 1.

The term "substantially corresponding" as used herein in relation to the nucleotide sequence encoding tomato ADH2 or a fruit ripening and/or aroma/flavour-affecting portion thereof, is intended to encompass minor variations in the DNA sequence which due to degeneracy in the DNA code do not result in a significant change in the encoded protein. Further this term is intended to encompass other

minor variations in the sequence which may be required to enhance expression in a particular system but in which the variations do not result in a decrease in biological activity of the encoded protein.

Preferably, the isolated DNA molecule according to the fourth aspect encodes an ADH2 or a fruit ripening and/or aroma/flavour-affecting portion thereof that is normally expressed during natural fruit ripening and further comprise a suitable constitutive promoter (e.g. CaMV 35 S promoter) or an inducible promoter, such as the promoter according to any one of the first to third aspects of the invention, or endopolygalacturonase, 1-aminocyclopropane-1-carboxylic acid oxidase or E8 promoters, expressibly linked to the ADH2 encoding sequence.

The ADH2 encoding sequence or a portion thereof (e.g. a 20-50 nucleotide portion) may be linked to the promoter in the opposite orientation for expression in a 3' to 5' direction such that antisense RNA is produced. By further including a nucleotide sequence encoding a catalytic domain, ribozymes targetted against ADH2 mRNA's may also be produced. Further, DNA molecules according to the fourth aspect of the invention may include enhancer elements or multiple copies of the anaerobic response element.

In a further aspect, the present invention provides a plant transformed with at least one DNA molecule according to any one of the first, second, third or fourth aspects.

In some applications, it may be preferred to transform the plant with multiple copies of a DNA molecule according to any one of the first, second, third or fourth aspects.

Preferably the DNA molecule(s) is stably inserted into the plant genome and will be transferred, via the seed or by clonal propogation, to subsequent generations.

Transformation may be via, for example, Agrobacterium tumefaciens-mediated transfer or the DNA particle gun method.

The invention thus permits suitable "slow ripening" or "slow softening" soft fruit cultivars to be transformed with hybrid DNA molecules to enable the production of wall loosening, colour-affecting and/or aroma/flavour enhancing enzymes. Where an inducible promoter is used, production of the enzyme(s) can be controllably regulated by, for example, selected temperature or gaseous treatments applied at a late stage in the fruit distribution and marketing chain.

In a specific case, benefits can be envisaged in modifying a slow-ripening tomato cultivar by incorporation of a hybrid DNA molecule comprising the ADH2 promoter from tomato, suitable enhancers, and the tomato polygalacturonase gene or ADH2 gene. During the early stages of distribution after harvesting advantage could be taken of the cultivar's inherent resistance to physical damage, but the promoter could be activated, at a point close to sale, to bring out a flavour akin to that of a fast ripening cultivar.

Northern analysis of ripening fruit of two tomato cultivars has shown that the mRNA for ADH2 is present in low abundance in mature green fruit and increases in abundance through ripening and, particularly, late in ripening. In fruit pericarp tissue exposed to atmospheres with 3% (v/v) oxygen, the ADH2 mRNA level increases to a maximum within 8-16 hours, and returns to the basal level within 16 hours of return to air. The mRNA level was sensitive to the oxygen level in the atmosphere, increasing 20 fold in 12% (v/v) oxygen and 100 fold in 3% oxygen. These oxygen levels may reflect appropriate levels for the induction of some inducible promoters according to the invention in commercial amounts of fruit

harvested from transgenic plants. Alternative methods for induction may include UV light, low temperatures (e.g. 0^{o}-10^{o}C) and exposure to some organic acids including gaseous CO_2.

Although, the inducible promoters envisaged by the invention are particularly described in relation to their expression in soft fruit, it should be appreciated that the promoters may be activated by the environmental agents and conditions in other tissues.

The invention will now be further described by way of the following, non-limiting examples and with reference to the accompanying figure.

Brief Description of Figures

Figure 1 is a diagram of the genomic clone lambda 2A3 indicating the location of exons 1 to 9 of the ADH2 gene, the relative positions of the EcoR1 fragments and the 5' region comprising the promoter. The region shown dashed has not been sequenced.

Example 1: Isolation of a cDNA encoding Tomato ADH2

Poly A^+ RNA was isolated from pericarp tissue of tomato (cv. de Ruiter 83G38) fruit, 9 days after the first appearance of colour (Breaker + 9). A cDNA library was constructed from the poly A^+ RNA and was cloned into lambda Gem 11 using the protocol of Promega Ltd. The library was screened by hybridisation with a ^{32}p-labelled fragment of tomato genomic DNA with sequence homology to ADH genes of pea (Llewellyn et al. J. Mol.Bio. 195:115-123, 1987) potato (Matton et al., Plant Mol.Biol. 14: 775-783, 1990) and maize (Dennis et al. Nucleic Acids Res. 13: 727-743, 1985). Four positive colonies were isolated, phage DNA was purified and cDNA inserts were transferred from the phage vector to a plasmid vector pGem 11 (Promega) for sequencing. Sequencing was carried out by the dideoxy-method of Sanger et al. PNAS, USA 74: 5463-5467 (1977) on double stranded

DNA using pUC universal and reverse primers and oligonucleotide primers to extend known sequence. The four cDNAs had identical 5' sequences and identical open reading frames encoding a protein of 364 amino acids.

The cDNA hybridised strongly to a 1.6 kb RNA species in RNA from ripe but not from unripe tomato fruit. Similarly, the cDNA hybridised to RNA from tomato seedling roots kept anaerobic overnight, but not to RNA from aerobic roots. In contrast, a Bgl11 fragment of tomato genomic cDNA, which contains regions encoding the tomato ADH1 isozyme and hybridises strongly to ADH1 mRNA but weakly to ADH2 mRNA (Wisman et al. Mol. & Gen. Genet. 226:120-128, 1991), did not hybridise to RNA from either ripe fruit or anaerobic root tissue. Thus it was concluded that the cDNAs isolated from the ripening fruit library, encode a tomato ADH2 enzyme.

The nucleotide sequence for the cDNA encoding the ADH2 mRNA is provided at Table 1.

The 5' 843 nucleotides of the cDNA including sequence encoding 250 amino acids of the ripening associated ADH2 enzyme provides a particularly useful probe by virtue of its location adjacent to the promoter in the genomic sequence.

Example 2: Isolation of Tomato ADH2 Promoter

Isolation of Tomato Leaf DNA.

Young leaves of tomato plants (cv. de Ruiter 83G38) were harvested and snap-frozen in liquid nitrogen. The leaves were ground to a fine powder under liquid nitrogen and DNA was isolated by the method of Thomas et al. Theor. Appl. Genetics 86: 173-180 (1993).

Construction of Genomic Library.

Aliquots of high molecular weight tomato genomic DNA were digested with varying amounts of Mbol restriction endonuclease for 1 hour at 37^{O}C and digestion was stopped by heating the samples at 75^{O}C for 10 minutes.

The samples were fractionated on a 0.5% agarose gel and the mean size and spread of the digested DNA was determined. 20μg of genomic DNA as digested with MboI enzyme, under conditions designed to give a fragment size range of between 10 kb and 20 kb, and the reaction was stopped as before. The digested fragments were partially end-filled with dATP and dGTP and were ligated into Lambda GEM-11 XhoI Half-site arms (Promega Corp.), according to the Promega protocols. Three ligations were carried out with arms:fragment ratios of: 0.5μg:0.25μg; 0.5μg:0.5μg; 0.5μg:0.75μg.

The ligated DNA samples were packaged according to Hohn, Methods in Enzymology 68:299-309 (1979) and titred, giving average titres of 1.8 x 10^5 pfu/μg arms. The three libraries were pooled.

Library Screening.

E. coli, strain KW251 cells, were infected with 2 x 10^5 phage, were spread on six, 10cm x 10cm Luria agar plates (33,000 pfu/plate) and were incubated at 37°C for 6 hours until small phage plaques had developed. Lifts were taken off the plates onto Biotrace NT nitrocellulose membranes (Gelman Sciences), and the membranes were prehybridised, hybridised and washed according to the Gelman protocols. Dried membranes were exposed to X-ray film (Fuji, RX) with an intensifying screen (Du Pont - Cronex, Lightning-Plus) at -70°C. Hybridisation probes were labelled with ^{32}p dATP by oligopriming (Feinberg and Voglestein, Anal.Biochem 132: 6-13, 1983).

Subcloning and Sequencing.

The genomic DNA insert of lambda clone 2A3 was digested with EcoR1 restriction enzyme and the resulting fragments subcloned by ligation into the plasmid vector pUC18 (Yanisch-Peron et al., Gene 33: 103-119, 1985) by standard methods.

Double stranded sequencing was carried out by the

enzymatic chain-termination method of Sanger et al. Proc.Natl.Acad.Sci. USA 74: 5463-5467 (1977), using universal and reverse M13 primers based on determined sequence.

PCR amplification of the 5' region of the ADH2 gene was carried out on a tomato genomic DNA template using oligonucleotide primers to regions of the AHD2 cDNA as follows:-

5' Primer #1078 5' CCACTGCCTCAACTGAG 3'

3' Primer #1057 3' CGACCATTCGGTAATCA 5'

PCR reactions were carried out in 50μl reactions containing 16.6mM $(NH)_4SO_4$, 67mM Tris-HCl pH 8.8, 6.7mM EDTA, 2mM $MgCl_2$, 0.15% Triton X-100, 200 μM dNTPs and 200μg/μl gelatin, with primers added at 22 pmol/μl, 100ng tomato genomic DNA and 2.5 units of Taq polymerase. Reactions were carried out as follows:

1st cycle: 94°C 5 mins.
45°C 1 min.
72°C 1 min.

Followed by 38 cycles as follows:

94°C 1 min.
45°C 1 min.
72°C 1.5 mins.

The PCR product was ligated into pBluescript KS^+ vector (Stratagene) linearised at the EcoRV site and T-tailed according to the procedure of Marchuk et al. Nucleic Acids Research 19.1154 (1990).

Results

Duplicate lifts were taken of six plates containing a total of 2 x 10^5 pfu of a phage library of tomato genomic DNA. The lifts were screened by hydridisation with two probes. The first probe, pADHCR1 was designed to be specific to the 5' end of the tomato AHD2 gene

which is expressed in ripening fruit. pADHCR1 was generated by PCR amplification of the genomic sequence bounded by primers #1078 and #1057 defined by the sequence of the ADH2 cDNA and spans exon 1, intron 1 and 34 nucleotides of exon 2 of the ADH2 gene. The second probe, pADH2-3' constitutes the entire ADH2 cDNA 3' of the EcoR1 site at nucleotide 290 on the cDNA (nucleotide 382 on the genomic DNA - Table 1).

Screening with the 5' specific probe, pADHCR1, gave 28 positives. Screening with the 3' specific probe, pADH2-3' gave 20 positives. Only one lambda phage plaque, 2A3, hybridised with both probes, and this plaque was isolated and purified for further characterisation.

EcoR1 digestion of DNA from the lambda genomic clone, 2A3, generated a number of fragments including the left and right arms of the lambda vector and three fragments of insert DNA with sizes of 7.8 kb, 1.4 kb and 1.2 kb. Subsequent subcloning revealed a further insert fragment of 63 bp in size. The EcoR1 fragments were subcloned into pUC18 and were sequenced, allowing alignment of the fragments to be determined (Fig. 1). The 1.4 kb subclone, pADH2-1.4 was found to contain the 5' end of the ADH2 gene, from the EcoR1 site at nucleotide 290 in the cDNA and including introns 1 and 2. The clone includes the transcription start site and extends a further 800 bp upstream encompassing the gene promoter.

The sequence of the clone 2A3 including the ADH2 promoter and the protein-encoding region (including introns) is provided at Table 1. The ADH2 gene has an overal length of 2334 bp from transcription site to poly A addition site.

Two regions, with a core sequence of 5'-AAACAA-3', showing homology to anaerobic response elements found in other dicots, are arranged in tandem upstream and close to

the putative transcription start site, between positions -313 and -240. One inducible promoter portion of the ADH2 promoter may therefore comprise these regions, e.g. residues -350 to -1 of Table 1.

Example 3: Production of Transformed Tomato Plants Expressing ADH2

The cDNA for tomato ADH2 described in Example 1 may be coupled in a normal orientation to the constitutive plant promoter CaMV 35S promoter. The chimeric gene may then be introduced into a suitable expression vector, which is subsequently used to transform tomato cells by any of the methods commonly known in the art.

Transformed plants may be analysed for the presence of the introduced ADH2 construct and for its expression. Correct synthesis of ADH2 enzyme will be checked by expression in bacterial cells transformed with the construct.

Positive plants (T0) will be selfed and homozygous transformants (T1) will be selected. ADH2 mRNA levels and ADH2 enzyme activity will be analysed in fruit from T1 plants.

Finally, volatile components of fruit will be analysed and fruits will be subjected to sensory evaluation.

By a similar method, transformed tomato plants may be produced which show a decreased expression of ADH2 in ripening fruit.

In this case however, the ADH2 cDNA shall be linked to the promoter in reverse orientation such that antisense RNA shall be produced.

Example 4: Production of Transformed Plants Expressing Polypeptide-Encoding DNA Sequences Using the ADH2 Promoter

The tomato ADH2 promoter described in Example 2 may be coupled to a nucleotide sequence encoding a polypeptide such as the tomato polygalacturonase enzyme. The chimeric

gene may then be introduced into a suitable expression vector, which can be subsequently used to transform tomato cells.

Expression of the introduced gene in transformed plants may be allowed to occur naturally during fruit ripening or may be induced by exposure of plant tissue to, for example, reduced oxygen atmospheres.

TABLE 1.

```
Genomic                                    GATCAAAAGATGAACTAAGGATAATATTTTTGATATTTTACCTTTTTTTTTAATTATAACGTTGTTAAGATATCAAAGGACCACTATTATAAGAAAACCACCC  -842

Genomic  AAAGGTTTCATGTTTTGATTGAAAAACCATCAAACAAGCCATACAACTCGCTCAACTAGAATCAAGCTAACAAAAAAATATACTTAATGAGATCATTTATAAGCTA  -735

Genomic  AATTATAACCCCTCCGTGCACTTTCATTTATCATGTTATATTTTACGCAAGTCAATTTCATTTTAAAGTTAAATGAGATTATATTATTTTAAT  -628

Genomic  AAAATTTTCAGATATTTAAAATTATATTGAAAGTATCATGAATTGTAGTTTTTTTTTTTGCATATATGAAAAATACATTATAAATATTAGTCAATTTTTTTATAATT  -521

Genomic  TGACTCTAAATATGAAAAAAAATGACAATTAAAAATAGACGGAGGTTGTAAATTAGCTTATTAATTATTAATTTGATAAATATCATAATTAACTGATAAATGACAATT  -414

Genomic  AAATATTTAGAAGACGATATGACAGAATCAACGTTATTTTAGGTATAATTTTTGTATTTTTTGAAAAAATAATCTTTTTTTCTGCAACTGGTTATATTAAGTG  -307
                                                                                                    * transcription start

Genomic  AACAACAAAAACAAGTAGTATAAAAAAATTACAAGTGGACATAAAACAAAATGAGATACAGTATTTGTGTTTCCATTGGATATTAGCTTGACAAAACTCAAAC  -200

Genomic  GAGCAGCAAACAAACAGCTAAAAAACCTGTTTTGAAAATCCAGTGACCAAAACATGTAAATGGTTTTACTGTGGCCTATTGTTTTTTTCACCTTTCCCAATTAT  -93

                                                                                                    1|M  S  T  T  V
cDNA     AAATATCCACTGCCTCAACTGAGTAAACAACCAAATTGTGTTCTATAAAAGTTTTCATATTTAGTGATCACTAAAAAAAAATCAAGAAGATGTCCGACTACTGTA  15
Genomic  AAATATCCACTGCCTCAACTGAGTAAACAACCAAATTGTGTTCTATAAAAGTTTTCATATTTAGTGATCACTAAAAAAAAATCAAGAAGATGTCCGACTACTGTA
                                                                                                    -GA1693

cDNA     GGCCAAGTCATTCGTTGCAAAG                    Intron 1                    |A  G  Q  V  I  R  C  K  |
                                                                                                    C
Genomic  GGCCAAGTCATTCGTTGCAAAGGTATATAATAATTCCATGATTCTGTAATTTCCTCGTTTTTTTTTTTTAAGTTTGATAATTTTTGTGGTAATTATATATTATTTATAGC  122

         A  V  A  W  E  A  G  K  P  L  V  H  E  E  V  D  V  A  P  P  Q  K  H  E  V  R  L  K  I  L  Y  T  S  L  C
cDNA     TGCTGTGGCATGGGAAGCTGGTAAGCCATTAGTGATGGAGGAAGTGGATGTTGCTCCTCCACAGAAATGGAAGTTCGTCTTAAGATCCTCTACACTTCACTCTGTC  -307
Genomic  TGCTGTGGCATGGGAAGCTGGTAAGCCATTAGTGATGGAGGAAGTAGATGTTGCTCCTCCACAGAAATGGAAGTTCGTCTTAAGATCCTCTATACTTCACTCTGTC  239

         H  T  D  V  Y  F  W  E  A  K  |
cDNA     ATACTGATGTATACTTCTGGGAAGCTAAG                    Intron 2
Genomic  ATACTGATGTATACTTCTGGGAAGCTAAGGTAAACAAAACTAAATTACGGGACTAGTTGAGTATGTTAGTGTTGTCAGCAAATTTTATAAGGGATTATTTCCTTT  346

                  |  G  Q  N  P  V  F  P  R  I  L  G  H  E  E  A  A  G|
cDNA                 GGTCAAAATCCAGTCTTTCCTCGAATTCTTGGACATGAAGCAGGAG                    Intron 3
Genomic  GAACTGATTTCAGGGTCAAAATCCAGTCTTTCCTCGAATTCTTGGACATGAAGCAGGAGGTATGTGTTATCTTGTTTCAATTGATTTGAATTCATCATTTAC  453

                     |  I  V  E  S  V  G  E  G  V  T  D  L  A  P  G  D  H  V
cDNA                    GATTGTGGAGAGTCTTGGAGAGGGAGTAACAGACCTTGCACCAGGAGACCATGTTC
Genomic  TGTTTCTAAAGCTAAAAGGGTACTGAATTTTGTTGTCTTCTTGATATTTAGGATTGTGGAGAGGGAGTAACAGACCTTGCACCAGGAGACCATGTTC  560

         L  P  V  F  T  G  E  C  K  D  C  A  H  C  K  S  E  E  S  N  H  C  S  L  L  R  I  N  T  D  R  G  V  H  L  N
cDNA     TTCCTGTCTTTACAGGGGAATGTAAAGATTGCGCCTCACTGTAAATCTGAAGAAAGCAATATGTGTAGCCTCTTAAGGATTAACACTGACAGGGGAGTGATCCTTAAT
Genomic  TTCCTGTCTTTACAGGGGAATGTAAAGATTGCGCCTCACTGTAAATCTGAAGAAAGCAATATGTGTAGCCTCTTAAGGATTAACACTGACAGGGGAGTGATCCTTAAT  667

         D  G  K  S  R  F  S  I  N  G  N  P  I  Y  H  F  V  G  T  S  T  F  S  E  Y  T  V  V  H  V  G  C  V  A  K  I
cDNA     GATGGAAAATCAAGATTTTCCATCAATGGAAACCCCATTTACCATTTTGTTGGGACCTCTACTTTTAGTGAGTACACCGTGGTTCATGTTGGATGTGTTGCAAAAT
Genomic  GATGGAAAATCAAGATTTTCCATCAATGGAAACCCCATTTACCATTTTGTTGGGACCTCTACTTTTAGTGAGTACACCGTGGTTCATGTTGGATGTGTTGCAAAAT  774

         N  P  L  A  P  L  D  K  V  C  V  L  S  C  G  I  S  T  G|
cDNA     TAACCCTCTTGCTCCTCTTGACAAAGTATGTGTCCTTAGTTGTGTGGAATTTCGACAGG                    Intron 4
Genomic  TAACCCTCTTGCTCCTCTTGACAAAGTATGTGTCCTTAGTTGTGTGGAATTTCGACAGGTATAGACGAAGACAACGATAGATTATGTTACTAGTTTCTTTTTAAGGAGC  881
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TABLE 1 continued.

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                                               | L  G  A  S  L  N  V  A  K  P  T  K  G  S  S  V  A  I  F  G  L  G  A
cDNA                                            CCTTGGAGCAAGTTTGAATGTTGCTAAACCAACAAAAGGCTCAAGTGTGGCTATATTTGGACTAGGAGCTG
Genomic  TGCTCAATTGTTGATTGATATGAATACTTTTCCAGGCCTTGGAGCAAGTTTGAATGTTGCTAAACCAACAAAAGGCTCAAGTGTGGCTATATTTGGACTAGGAGCTG   988

         V  G  L  A |                         Intron 5                                         | A  A  E  G  A  R
cDNA     TAGGCCTCGCG                                                                            GCTGCAGAAGGAGCCAGA
Genomic  TAGGCCTCGCGGTGAGTATGCTCCGTTGTGTTGTTTTATTGTTTCCCGTATATGTGTTAGTCTTACAGATGACTGACTCATTTGGTCAGGCTGCAGAAGGAGCCAGA  1095

          I  A  G  A  S  R  I  I  G  V  D  L  N  A  S  R  F  E  Q |                               Intron 6
cDNA     ATTGCTGGTGCCTCGAGGATAATTGGTGTTGATTTAAATGCTAGTAGATTTGAGCAAG
Genomic  ATTGCTGGTGCCTCGAGGATAATTGGTGTTGATTTAAATGCTAGTAGATTTGAGCAAGGTAATATAAATTTTTCCTTATACATTATCTTAAAATTCCTTAGTAAAAC  1202

                                      |A  K  K  F  G  V  T  E  F  V  N  P  K  D  Y  S  K  P  V  Q  E |
cDNA                                   CTAAGAAATTTGGTGTGACAGAGTTTGTGAACCCAAAGGACTATAGTAAACCAGTTCAAGAG
Genomic  AACTAATTCATCCATTTTACTTGTATTCTACAGCTAAGAAATTTGGTGTGACAGAGTTTGTGAACCCAAAGGACTATAGTAAACCAGTTCAAGAGGTACTCAAATCA  1309

                           Intron 7                                                         | V  I  A  E  M  T  D
cDNA                                                                                          GTAATTGCTGAGATGACTGA
Genomic  TATTTAATTTACTTTAATCGAAGAAGAAAAAAGACAGGTCTGAGTTAATAGTTGATGTCTTTTCTTGAATTCTGATTATTTGATCAGGTAATTGCTGAGATGACTGA  1416

          G  G  V  D  R  S  V  E  C  T  G  H  I  D  A  M  I  S  A  F  E  C  V  H  D |                  Intron 8
cDNA     TGGCGGAGTCGATAGGAGTGTGGAATGTACTGGTCACATTGATGCTATGATTTCAGCATTTGAATGTGTCCATGATG
Genomic  TGGCGGAGTCGATAGGAGTGTGGAATGTACGGGTCACATTGATGCTATGATTTCAGCATTTGAATGTGTCCATGATGTATGTTTTCTGTAATCAAATTAATTTCCTT  1523

                                                        |G  W  G  V  A  V  L  V  G  V  P  H  K  E  A  V  F  K  T
cDNA                                                     GCTGGGGAGTGGCGGTTCTTGTTGGTGTACCCCATAAAGAAGCTGTGTTCAAGACAC
Genomic  AGCTGTATGTTTGCGTTCATCTTAACGAACATTGTTGTATTAACTTTAGGGCTGGGGAGTCGCGGTTCTTGTTGGTGTACCCCATAAAGAAGCTGTGTTCAAGACAC  1630

          H  P  L  N  F  L  N  E  R  T  L  K  G  T  F  F  G  N  Y  K  P  R  S  D  I  P  C  V  V  E  K  Y  M  N  K  E
cDNA     ATCCTCTGAACTTTTTGAATGAACGGACTCTCAAAGGAACCTTCTTTGGAAACTACAAACCTCGTTCGGATATTCCTTGTGTTGTTGAGAAATACATGAACAAAGAA
Genomic  ATCCTCTGAACTTTTTGAATGAACGGACTCTCAAAGGAACCTTCTTTGGAAACTACAAACCTCGTTCGGATATTCCTTGTGTTGTTGAGAAATACATGAACAAAGAA  1737

          L  E  L  E  K  F  I  T  H  T  L  P  F  A  E  I  N  K  A  F  D  L  M  L  K  G  E  G  L  R  C  I  I  T  M  A
cDNA     CTTGAATTGGAGAAATTCATCACTCATACACTTCCATTTGCTGAAATCAATAAGGCTTTCGATTTAATGCTGAAGGGAGAAGGCCTTCGTTGCATCATCACCATGGC
Genomic  CTTGAATTGGAGAAATTCATCACTCATACACTTCCATTTGCTGAAATCAATAAGGCTTTCGATTTAATGCTGAAGGGAGAAGGCCTTCGTTGCATCATCACCATGGC  1844

          D  *
cDNA     GGACTAAACTTTCTGTCCTAGAAAAGGAGCTTCTACTGTTTGAGAAAAAAGACCAATAAATTGTCACTGTCTTATTTTCCCTTTCGTGTTTGGTTGAGTTGTAACAT
Genomic  GGACTAAACTTTCTGTCCTAGAAAAGGAGCTTCTACTGTTTGAGAAAAAAGACCAATAAATTGTCACTGTCTTATTTTCCCTTTCGTGTTTGGTTGAGTTGTAACAT  1951

cDNA     TCCATCCATGTCTCTTCTTTTGTCTTTTGCTTAGATGTTGTGCTTTGCCATATCTCTTTCGATTCTTGTAAAAAATGCAAATTCTCTCTGTTTTATCTCAAGTATAT
Genomic  TCCATCCATGTCTCTTCTTTTGTCTTTTGCTTAGATGTTGTGCTTTGCCATATCTCTTTCGATTCTTGTAAAAAATGCAAATTCTCTCTGTTTTATCTCAAGTATAT  2058

cDNA     TTACAGAATTTCAGTGATTTGATAAATCTAAACTTTATCATAATATAATCCAAACAGAATTTCAATTGAAAAAAA
Genomic  TTACAGAATTTCAGTGATTTGATAAATCTAAACTTTATCATAATATAATCCAAACAGAATTTCAATTGAAAATGATGAAGCCCTTACCGTCATTGTTCC  2175
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It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

CLAIMS:

1. An isolated DNA molecule comprising a nucleotide sequence encoding a soft fruit promoter or functional portion thereof, wherein said promoter or functional portion thereof is characterised in that it can be activated by environmental agents or conditions and/or is activated, or primarily activated, during a late stage of normal soft fruit ripening.

2. An isolated DNA molecule according to claim 1, wherein the activating environmental agents or conditions are selected from the group consisting of low temperature, UV light, or exposure to particular levels of oxygen, carbon dioxide, carbon monoxide or organic acids.

3. An isolated DNA molecule according to claim 2 wherein the activating environmental agents or conditions is low temperature.

4. An isolated DNA molecule according to claim 2, wherein the activating environmental agents or conditions is exposure to UV light.

5. An isolated DNA molecule according to claim 2, wherein the activating environmental agents or conditions is exposure to particular levels of oxygen, carbon dioxide, or carbon monoxide.

6. An isolated DNA molecule according to any one of the preceding claims, wherein the soft fruit promoter or functional portion thereof is from grapes, strawberries, peaches, plums or tomatoes.

7. An isolated DNA molecule according to any one of the preceding claims, further characterised in that it is activated by the environmental agents or conditions and is also activated, or primarily activated, during a late stage of normal soft fruit ripening.

8. An isolated DNA molecule comprising a nucleotide sequence encoding, or substantially homologous to, the tomato ADH2 promoter or a functional portion thereof.

9. An isolated DNA molecule comprising a nucleotide sequence substantially corresponding to that shown in Table 1 from residue -942 to -1.

10. An isolated DNA molecule comprising a nucleotide sequence encoding a plant promoter obtained from a genomic fragment isolated using a labelled nucleic acid probe comprising a nucleotide sequence substantially corresponding to the cDNA sequence or a portion thereof shown in Table 1.

11. An isolated DNA molecule according to any one of the preceding claims, further comprising a nucleotide sequence encoding a peptide or polypeptide expressively linked to the promoter sequence.

12. An isolated DNA molecule according to claim 11, wherein the peptide or polypeptide is involved in fruit softening, flavour, colour or aroma.

13. An isolated DNA molecule according to claim 12, wherein the peptide or polypeptide is selected from the group consisting of polygalacturonase or its sub-units, pectin methyl esterase, xyloglucanase or other β-1,4-glucanases, glycosidases, β-galactosidase, alcohol dehydrogenase and lipoxygenase.

14. An isolated DNA molecule according to claim 11, wherein the peptide or polypeptide is a fungal resistance agent or other plant pathogen resistance agent.

15. An isolated DNA molecule according to claim 14, wherein the peptide or polypeptide is chitinase or β-1,3-glucanase.

16. An isolated DNA molecule comprising at least a 20 nucleotide portion of a nucleotide sequence encoding a peptide or polypeptide, wherein the at least 20 nucleotide portion is linked to a promoter sequence as provided by a DNA molecule according to any one of claims 1-10, in the opposite orientation for expression in a 3' to 5' direction such that antisense RNA is produced.

17. An isolated DNA molecule according to claim 16, further comprising a nucleotide sequence encoding a catalytic domain such that expression results in the production of ribozymes for cleaving mRNA's encoding the peptide or polypeptide.

18. An isolated DNA molecule comprising a nucleotide sequence encoding tomato ADH2 or a fruit ripening and/or aroma/flavour-affecting portion thereof.

19. An isolated DNA molecule according to claim 18 comprising a nucleotide sequence substantially corresponding to the cDNA sequence shown in Table 1 or the genomic sequence from residue 1-2175 shown in Table 1.

20. An isolated DNA molecule according to claim 19 wherein the ADH2 or a fruit ripening and/or aroma/flavour effecting portion thereof is normally expressed during natural fruit ripening.

21. An isolated DNA molecule according to any one of claims 18-20, further comprising an operably linked constitutive promoter or inducible promoter.

22. An isolated DNA molecule according to claim 21, wherein the operably linked promoter sequence is encoded by an isolated DNA molecule according to any one of claims 1-10 or is selected from the group consisting of the CaMV 35S promoter, the endopolygalacturonase promoter, 1-aminocyclopropane-1-carboxylic acid oxidase promoter or E8 promoter.

23. An isolated DNA molecule comprising of at least a 20 nucleotide portion of the ADH2-encoding sequence as provided by a DNA molecule according to any one of claims 18 to 20, wherein the at least 20 nucleotide portion is linked to a constitutive or inducible promoter sequence in the opposite orientation for expression in a 3' to 5' direction such that antisense RNA is produced.

24. An isolated DNA molecule according to claim 23, further comprising a nucleotide sequence encoding a

catalytic domain such that expression results in the production of ribozymes for cleaving mRNA's encoding ADH2.

25. A plant transformed with at least one DNA molecule according to any one of the preceding claims.

26. A plant according to claim 25, wherein the plant has been transformed with multiple copies of a DNA molecule according to any one of the preceding claims.

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FIGURE 1